Answers to these issues do not simply interest basic scientists because clinical applications depend heavily on knowledge of RME. For example, Wilson et al. (10) have used the highly efficient hepatic uptake of ASOR for liver-directed gene therapy, whereby exogenous DNA linked to ASOR is endocytosed and in some hepatocytes incorporated into the genome. How DNA is sometimes spared lysosomal degradation is as yet unknown; enhancing the efficiency of endocytic delivery to the nucleus will clearly increase the effectiveness of this therapy. A second example is targeted drug delivery. By linking cytotoxic agents, such as diphtheria toxin, to tissue-specific ligands to generate "magic bullets," cytoreductive treatment of malignancies arising from that tissue can be enhanced. Understanding the events regulating segregation of cytotoxic ligand from the receptor and, in this case, entry of the toxin or drug into the cytoplasmic space will facilitate the design of more effective therapeutic agents. Third, RME plays a key role in the pathological uptake of selected viruses and microbiological toxins. A better understanding of endocytic sorting events may enable the development of inhibitors of the infectious process. The current study adds to the substantial insights into the push-and-pull of endocytic sorting, which have been gained in only a few recent years. Further advances in our understanding of intracellular membrane trafficking are eagerly anticipated.

JAMES J. CRAWFORD, M.D., PH.D.
Department of Pathology
Brigham and Women's Hospital and
Harvard Medical School
Boston, MA 02115

REFERENCES

1. Burwen SJ, Jones AL. Hepatocellular processing of endocytosed proteins. J Electron Microsc Tech 1990;14:140-151.
2. Schroer TA, Sheetz MP. Functions of microtubule-based motors. Annu Rev Physiol 1991;53:629-652.
3. De Brabander M, Nuydens R, Geerts H, Hopkins CR. Dynamic behavior of the transferrin receptor followed in living epidermoid carcinoma (A431) cells with nanovid microscopy. Cell Motil Cytoskeleton 1988;9:30-47.
4. Lin SXH, Collins CA. Immunolocalization of cytoplasmic dynein to lysosomes in cultured cells. J Cell Sci 1992;101:125-137.
5. Mueller SC, Hubbard AL. Receptor-mediated endocytosis of asialoglycoproteins by rat hepatocytes: receptor-positive and receptor-negative endosomes. J Cell Biol 1986;102:932-942.
6. Cooper MS, Cornell-Bell AH, Chernjavsky A, Dani JW, Smith SJ. Tubulovesicular processes emerge from *trans*-Golgi cisternae, extend along microtubules, and interlink adjacent *trans*-Golgi elements into a reticulum. Cell 1990;61:135-145.
7. Hollenbeck PJ, Swanson JA. Radial extension of macrophage tubular lysosomes supported by kinesin. Nature 1990;346: 864-866.
8. Van der Sluijs P, Hull M, Wabster P, Male P, Goud B, Mellman I. The small GTP-binding protein rab4 controls an early sorting event on the endocytic pathway. Cell 1992;70:729-740.
9. Schiff JM, Huling SL, Jones AL. Receptor-mediated uptake of asialoglycoprotein by the primate liver initiates both lysosomal and transcellular pathways. HEPATOLOGY 1986;6:837-847.
10. Wilson JM, Grossman M, Wu CH, Chowdhury NR, Wu GY, Chowdhury JR. Hepatocyte-directed gene transfer *in vivo* leads to transient improvement of hypercholesterolemia in low density lipoprotein–deficient rabbits. J Biol Chem 1992;267:963-967.

Acknowledgments: Appreciation is expressed to Peter J. Hollenbeck for his critical reading of this review. This review was supported by NIH grant DK3951.

THE SELF-SUSTAINED SEQUENCE REPLICATION AMPLIFICATION SYSTEM – MOVE OVER, PCR

Gingeras TR, Prodanovich P, Latimer T, Guatelli JC, Richman DD, Barringer KJ. Detection of human immunodeficiency virus type I in AIDS patients using amplification-mediated hybridization analyses: Reproducibility and quantitative limitations. J Infect Dis 1991;164:1066-1074.

ABSTRACT

Mutations at amino acid positions 67, 70, 215, and 219 in the human immunodeficiency virus type 1 (HIV-1) *pol* gene correlate with the emergence of resistance to zidovudine (AZT). These four positions were monitored in viral RNA extracted from infected peripheral blood mononuclear cells (PBMC) and viral stocks obtained after coculture with uninfected lymphocytes. Genotype determinations were made using the self-sustained sequence replication (3SR) and differential bead-based sandwich hybridization (BBSH) assay. The hybridization results obtained by 3SR and BBSH analyses were verified by dideoxynucleotide sequencing of the 3SR products. Correlation of 3SR and BBSH with the polymerase chain reaction and Southern hybridization analyses of the PBMC and corresponding viral isolates indicated that PBMC and corresponding HIV-1 isolates may differ in their genotypes at the monitored amino acid positions, variations from the wild-type nucleotide sequence may occur proximal to the codons being monitored, and viral isolates possessing the same genotypes at the four monitored amino acid positions showed a threefold variation in their ID_{50} measurements.

COMMENTS

The introduction of the polymerase chain reaction (PCR) revolutionized molecular biology research. PCR amplification consists of multiple cycles of DNA replication directed by target-specific oligonucleotide primers. Each cycle is separated by a short period of heating (92° to 97° C) to permit strand separation of the newly synthesized DNA for further replication. This powerful technique has enabled even nonmolecular biologists to detect, isolate and determine the nucleotide sequence of RNA or DNA of interest. PCR's ability to amplify minute quantities of a nucleic acid sequence has also made it useful in clinical medicine as a diagnostic tool – for example, to identify human immunodeficiency virus (HIV) or hepatitis C infection. However, PCR does have several inherent drawbacks. PCR requires the use of expensive thermal cycling equipment and heat-stable DNA polymerases. In addition, PCR amplification only works with DNA; thus any RNA sample must first be converted to complementary DNA in a separate step before it can be amplified by PCR. Finally, the use of PCR as a clinical diagnostic tool has been limited by complications related to patent rights. Recently, several

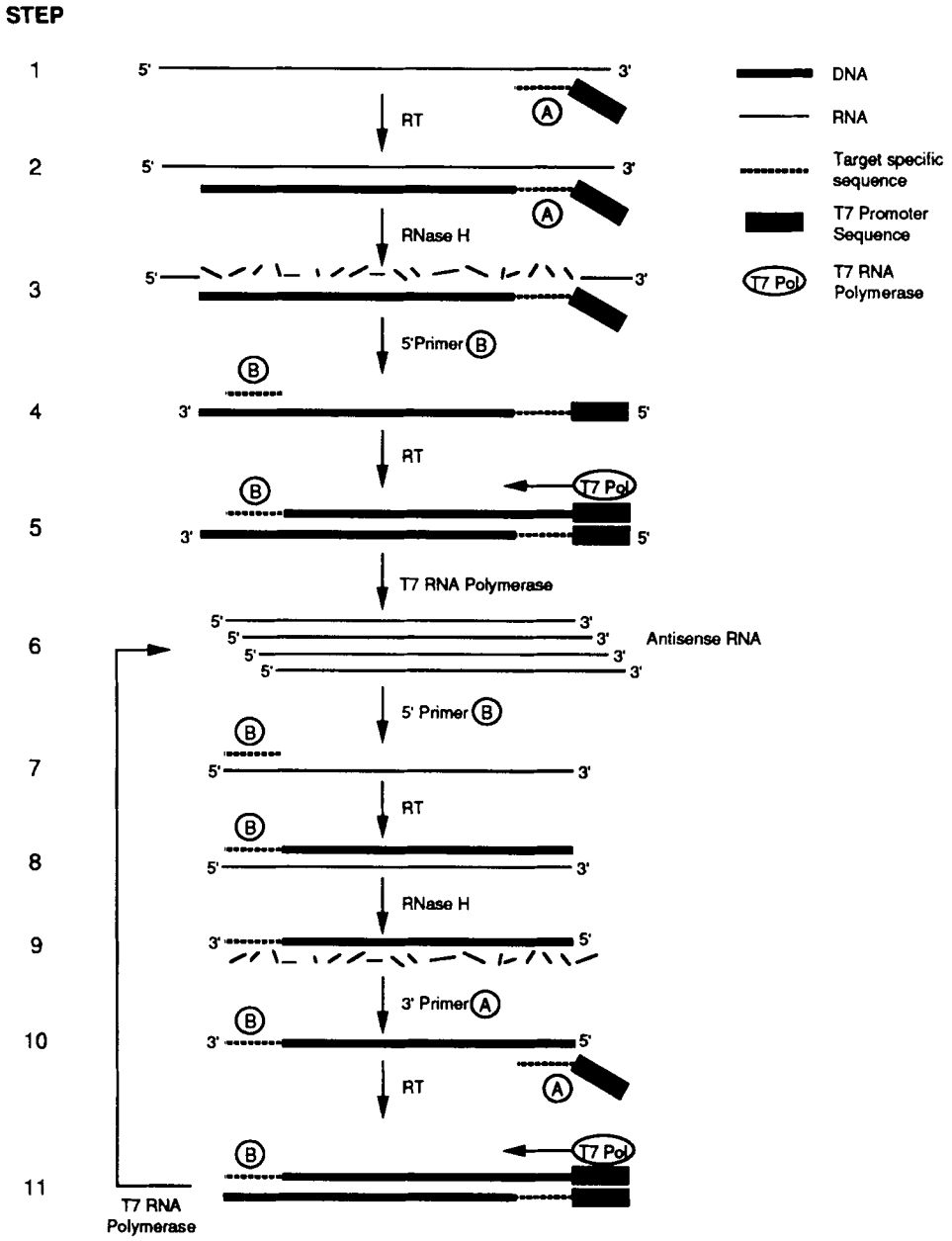

FIG. 1

isothermal amplification methods have been developed that not only avoid these problems but also have some unique advantages over PCR. One such technique is the self-sustained sequence replication (SSSR) reaction (1) used in this study by Gingeras et al. However, other systems including the Qβ replicase system (2), strand displacement amplification (3) and a modified strand displacement amplification (4) have also been described.

The SSSR reaction is a modification of the previously described transcription-based amplification system (5), which is a thermal cycling–based technique. Both of these systems are modeled after the general scheme employed during retrovirus replication. The ability of the SSSR reaction to proceed isothermally is dependent on the combined activity of three enzymes: avian myeloblastosis virus (AMV) reverse transcriptase, T7 RNA polymerase and ribonuclease H (RNase H).

The general strategy for SSSR amplification of an RNA target sequence is illustrated in Figure 1. After an initial denaturation of 65° C, AMV reverse transcriptase, RNase H, T7 RNA polymerase, dGTP, dATP, dTTP, dCTP, GTP, ATP, UTP, CTP and two synthetic oligonucleotide primers (referred to here as primer A and primer B) are added to the sample and incubated at 37° to 42° C for 1 hr. Primer A consists of 15 to 30 base pairs, which are complementary and specific for the 3′ end of the target sequence. Incorporated at the 5′ end of this primer are an additional 25 base pairs that contain the recognition and initiation site for T7 RNA polymerase. Primer B consists of 15 to 30 base pairs specific for the

5′ end of the target sequence, although it is possible to include a T7 RNA polymerase recognition sequence at the 5′ end of this primer as well.

As shown in Figure 1 *(step 1 and 2)*, the target specific sequence of primer A anneals to the RNA of interest and is extended as DNA by the activity of AMV reverse transcriptase. The RNA strand of this RNA-DNA duplex is subsequently degraded by the activity of RNase H *(step 3)*. Primer B is then able to anneal to the 3′ end of the resulting single-stranded DNA and is extended by the DNA-dependent DNA polymerase activity of AMV reverse transcriptase *(steps 4 and 5)*. This creates the double-stranded DNA recognition site required by T7 RNA polymerase. The actual amplification of the target sequence occurs in step 6 as T7 RNA polymerase generates 10 to 1,000 antisense RNA copies *(step 7)* from each double-stranded DNA template. Primer B anneals to each of these RNA copies and is extended as DNA by AMV reverse transcriptase *(steps 8 and 9)*. RNase H digests the RNA of the RNA-DNA duplex formed, and primer A anneals to the resulting single-stranded DNA *(steps 10 and 11)*. The DNA polymerase activity of AMV reverse transcriptase extends the primer A and the DNA strand containing primer B to full length to again create a double-stranded DNA containing the T7 RNA polymerase recognition site *(step 11)*. The antisense RNA copies generated are then available for subsequent cycles of DNA extension, RNA degradation and T7 RNA amplification *(steps 7 to 11)*. This protocol can also be modified to amplify a DNA target, as was done in this study. The reaction mixture, without enzymes, is heated at 100° C for 5 min and annealed at 42° C. AMV reverse transcriptase is added and incubated for 10 min (analogous to step 1) before a second denaturation and annealing step. This additional denaturation step produces the single-stranded DNA that would have been formed by the activity of RNase H during RNA amplification. All three enzymes are then added, and the reaction proceeds as described above from step 4.

Depending on the efficiency of the reaction, between 10^6-fold and 10^7-fold amplification of the target sequence is expected after a 1- to 2-hour incubation. The amplified product is primarily single-stranded antisense RNA, although small amounts of DNA-RNA hybrid and double-stranded DNA are also produced (1).

The SSSR reaction has several advantages over PCR. It does not require thermal cycling equipment and, unlike PCR, genomic DNA will not be inadvertently amplified in an attempt to amplify RNA. As in this article, the products of the SSSR reaction can also be used directly for sequencing (without subcloning) because they are primarily single stranded and antisense. The SSSR reaction can be used to generate results comparable to those yielded by PCR even in very complex applications, such as the one described in this article. However, by far the greatest advantage of the SSSR reaction over PCR is its ease of use. This makes it eminently practical for mass screening of samples for RNA or DNA of interest. One could perform a simple preparation to isolate DNA or RNA from patients' samples, heat them, add all of the necessary reagents and then incubate them for 1 or 2 hr. The resulting amplified product could then be detected with any number of existing methods, including the bead-based sandwich hybridization assay (5) used in this article. For example, SSSR could be very useful in screening serum samples for hepatitis C RNA or hepatitis B DNA. Because SSSR amplification is not a patented process, it could be adapted for use as a clinical diagnostic test as well.

Despite of all of these advantages, this method does have some disadvantages that are likely to keep it from replacing the PCR in many applications. The low temperature of the SSSR reaction may reduce the specificity of the oligonucleotide primers. Also, the length of the primers needed to include the target specific sequence and the T7 RNA polymerase recognition site make them expensive to synthesize. Finally, the length of the target segment that can be amplified is also limited in this system.

In summary, the SSSR system is a simple and powerful technique that may replace PCR in many applications and may be especially useful in screening large numbers of samples for RNA or DNA sequences of interest.

KENNETH S. LOWN, M.D.
Division of Gastroenterology
University of Michigan Medical Center
Ann Arbor, Michigan 48109

REFERENCES

1. Guatelli JC, Whitfield KM, Kwoh DY, Barringer KJ, Richman DD, Gingeras TR. Isothermal, in vitro amplification of nucleic acids by a multienzyme reaction modeled after retroviral replication. Proc Natl Acad Sci USA 1990;87:1874-1878.
2. Pritchard CG, Stefano JE. Amplified detection of viral nucleic acid at subattomole levels using Q beta replicase. Ann Biol Clin 1990;48:492-497.
3. Walker GT, Little MC, Nadeau JG, Shank DD. Isothermal *in vitro* amplification of DNA by a restriction enzyme/DNA polymerase system. Proc Natl Acad Sci USA 1992;89:392-396.
4. Walker GT, Fraiser MS, Schram JL, Little MC, Nadeau JG, Malinowski DP. Strand displacement amplification: an isothermal, *in vitro* DNA amplification technique. Nucleic Acids Res 1992;20:1691-1696.
5. Kwoh DY, Davis GR, Whitfield KM, Chappelle HL, DiMichele LJ, Gingeras TR. Transciption-based amplification system and detection of amplified human immunodeficiency virus type 1 with a bead-based sandwich hybridization format. Proc Natl Acad Sci USA 1989;86:1173-1177.

Reviews Elsewhere

Dingwall C, Laskey R. **The nuclear membrane.** Science 1992;258:942-947.

Nelson WJ. **Regulation of cell surface polarity from bacteria to mammals.** Science 1992;258: 948-955.

Luna EJ, Hitt AL. **Cytoskeleton-plasma membrane interactions.** Science 1992;258:955-964.

Morimoto RI, Sarge KD, Abravaya K. **Transcriptional regulation of heat shock genes.** J Biol Chem 1992;267:21987-21990.